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14. ABSTRACT
We hypothesized that the tightly regulated ratio of NF1 isoforms is critical in maintaining the homeostasis of cell growth and differentiation, thereby determining the functional output of NF1 gene. A corollary is that splicing regulators may modify the function of neurofibromin through altering alternative splicing of the NF1 pre-mRNA.
We propose two specific aims to study the role of regulated alternative splicing in the function of neurofibromin. In **Aim I,** we will determine how changes in alternative splicing affects NF1 function. In **Aim II**, we will determine the biological consequence of altering the ratio of neurofibromin isoforms in cells with natural NF1 expression such as neuronal and glial cells.
To this end, we have identified the cis-acting elements located to the vicinity of exon 23a that play key roles in regulating inclusion of this exon. To study the biological output of NF1 as a result of altered expression of splicing factors, we made lentiviruses that over-express Hu proteins. We also designed the gene-targeting strategy to engineer the NF1 locus in mouse ES cells and are in the process of carrying out the multiple steps of recombinant DNA cloning.
Results of the proposed studies will not only provide important novel insights into the etiology of NF1 disease, but also shed light on how genetic variations in splicing regulators affect the progression of other diseases.

15. SUBJECT TERMS
Alternative splicing, NF1 exon 23a, splicing signals, gene-targeting

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INTRODUCTION

We hypothesize that the tightly regulated ratio of NF1 isoforms is critical in maintaining the homeostasis of cell growth and differentiation and thus, it determines the functional output of NF1 gene. Furthermore, splicing regulators may modify the function of neurofibromin through altering alternative splicing of the NF1 pre-mRNA. There are two main goals of the proposed studies. We will first investigate how alternative splicing modulates the function of neurofibromin. Particularly, we will determine how cis-acting RNA sequence elements and trans-acting splicing regulators modulate the function of neurofibromin by examining not only the splicing status of exon 23a but also measuring the Ras activity, the functional output of altered NF1 splicing. The second goal of these studies is to determine the biological consequence of altered ratio of the two neurofibromin isoforms. We will create a unique system that will allow us to readily modify the NF1 alleles. We will use this system to generate mouse ES cell lines that express the two NF1 isoforms at different ratios. Subsequently, we will use a recently developed differentiation strategy to derive neural stem cells from these ES cells to examine the cell growth and differentiation potential of these cells.

BODY

During the last funding period, we made progress in three areas towards the goals of the two specific aims. First, we identified the point mutations at both acceptor and donor site of exon 23a, which will ensure predominant inclusion of this exon in all cell types (Aim Ia). These results laid the foundation for sequence engineering in mouse ES cells. Second, we generated lentiviral expression vectors of two Hu proteins and obtained viral stocks to be used in transduction assay (Aim Ib). Third, based on the information learned from Aim Ia, we designed the strategy to generate the knock-in allele that will be introduced into mouse ES cells. The strategy involves multiple steps of DNA cloning, some of which are very challenging. These experiments are currently in progress.

Aim I. To determine how altered alternative splicing affects NF1 function. Our preliminary data indicate that Hu proteins and TIAR are involved in the regulation of exon 23a inclusion. In **Aim Ia**, we will investigate the mechanism by which the cis-acting RNA regulatory sequence on NF1 pre-mRNA regulates inclusion of exon 23a. Multiple NF1 reporter constructs carrying mutations of key regulatory sequence elements will be generated and tested for splicing by transfecting two types of cultured cells, one that processes the NF1 pre-mRNA to predominantly include exon 23a, and the other that processes to predominantly exclude this exon. The results of these experiments will not only test the contribution of the cis-acting regulatory sequences to regulated alternative splicing, but also lay the foundation for the study in the Aim IIa. The goal is to create several mini-gene reporters that, in transfected cells, will be processed to have differing ratios of exon 23a+/exon 23a- ranging from predominantly exon 23 inclusion to predominantly exon 23 exclusion. **In Aim Ib**, we will change expression level of Hu proteins or TIAR and examine the function of neurofibromin. We will either over-express tissue culture cells that have little expression of Hu proteins or knockdown the expression of these proteins in cells that express these proteins and then determine how alternative splicing involving exon 23a is affected and how Ras activity output is affected.

Progress

1. Contribution of splice site strength to regulated inclusion of NF1 exon 23a (Aim Ia).

In general, alternatively included exons tend to have suboptimal splicing signals to allow regulation. To understand which the relative contribution of each of the splicing signals of exon 23a, we mutated these sequences individually and in combination in the NF1 reporter constructed generated in the laboratory as shown in Fig. 1. Fig. 2A indicates the mutations we created. The M1 mutant is designed to test how splicing signals contributed to the regulated inclusion of exon 23a. In M1A and M1D, the splicing acceptor and donor are mutated to their optimal sequence separately that will have the strongest potential to bind splicing factors. In particular, in M1A, the polypyrimidine tract sequence was mutated to the best U2AF binding sequence. In M1D, the 5' donor sequence was mutated to the perfect match to U1 snRNA. M1 combines these two mutations.

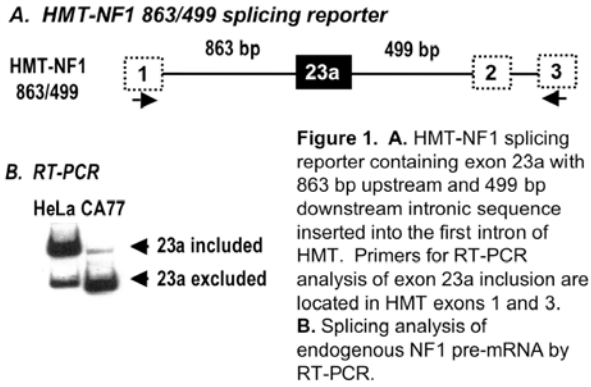

Figure 1. A. HMT-NF1 splicing reporter containing exon 23a with 863 bp upstream and 499 bp downstream intronic sequence inserted into the first intron of HMT. Primers for RT-PCR analysis of exon 23a inclusion are located in HMT exons 1 and 3. **B.** Splicing analysis of endogenous NF1 pre-mRNA by RT-PCR.

These mutant reporter constructs were transfected into two cultured cell lines that have been established in my laboratory and show the opposite outcome regarding inclusion of exon 23a of the endogenously expressed NF1 gene. HeLa cells process the endogenously expressed NF1 pre-mRNA to predominantly include exon 23a, while CA77 cells, a rat medullary thyroid carcinoma cell line that shows significant neuronal features, process the endogenously expressed NF1 pre-mRNA to predominantly exclude exon 23a (Fig. 1B). Splicing of NF1 pre-mRNA was determined by RT/PCR assay on total RNA isolated from cells transfected with the NF1 mutant reporters (as shown in Fig. 2A). As predicted, when the splice donor or/and acceptor was mutated to their consensus sequences, exon 23a inclusion was increased and this was specifically obvious in CA77 cells that process the wild type NF1 to exclude the exon (Fig. 2B).

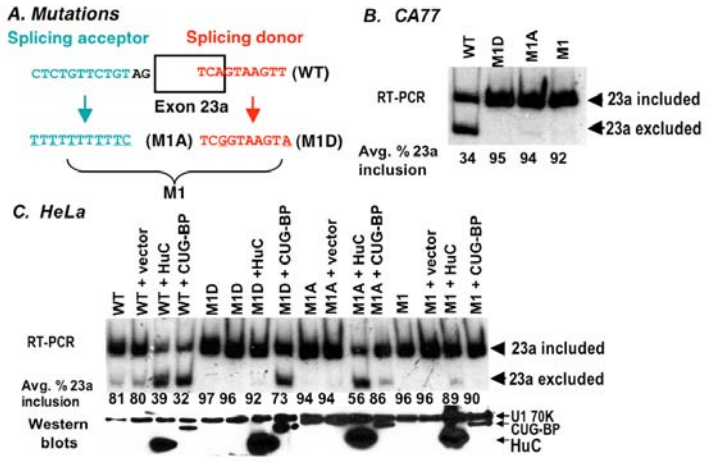

Figure 2. A. Point mutations gemerated. M1 contains mutation of both splicing donor and acceptor sequences. These changesare intended to strengthen the splice site signals **B.** Splicing of WT and mutant NF1 reporters in CA77, measured by RT-PCR. **C.** Splicing of WT and mutant NF1 reporters in HeLa, measured by RT-PC. Western blots showing overexpression of negative splicing regulators CUG-BP and HuC.

Because research in my laboratory demonstrated that both Hu proteins and CELF (CUG-BP and ETR3-like factors) proteins promote skipping of exon 23a of the NF1 pre-mRNA (Zhu et al., 2008 and unpublished data), we examined if these proteins could still regulate the mutant reporters. As shown in Fig. 2C, when co-transfected with HuC or CUG-BP cDNA plasmid, the wild type reporter showed strong response to the transfected proteins, while the mutants, especially the double mutant showed

little response. These results indicate that we have identified a way to engineer the NF1 sequence so the resulting pre-mRNA will predominantly include exon 23a in most cell types.

2. Effect of altered levels of trans-acting factors (Hu proteins or TIAR) on the function of the NF1 gene product

To determine if the biological output of the NF1 expression will be changed as a result alternative splicing change of exon 23a, we planned to alter expression of its regulators. We started with the Hu proteins. The relative low transfection efficiency makes it very difficult to assess the effect on over-expressed Hu protein on functional output of NF1, i.e., Ras signaling, we decided to use the lentiviral tranduction system to carry out this experiment. We cloned the mHuB and mHuC into a lentiviral vector, packaged the expression vectors into virus particles. The next step is to determine the titer of the virus and carry out transduction.

Aim II. **To determine the biological consequence of altered ratio of neurofibromin isoforms in cells with natural NF1 expression such as neuronal and glial cells.** In **Aim IIa**, to examine the NF1 function in its natural context, we will integrate the engineered sequences determined by Aim Ia that lead to differing ratios of exon 23a+/exon 23a- into the endogenous NF1 allele using gene targeting technology. An elegant state-of-the-art strategy will be used to obtain targeted ES cell lines with a series of different NF1 alleles. In **Aim IIb**, we will analyze the growth and differentiation potential of cells harboring the modified NF1 alleles. Neural stem cells (NTC) will be derived from these ES cells and analyzed for their growth potential in comparison to that derived from wild type ES cells using a recently described ES cell differentiation procedure.

Progress

Generate the targeting vector (Aim IIa)

We designed the strategy to create the targeting vector as outlined in Fig. 3A-C. First, a retrieval vector with short sequences of homology to NF1 will be created that will recombine in E. coli with an NF1-containing BAC to form the NF1 homology vector, which contains exon 23a along with ~2500 base pairs upstream and ~3000 base pairs downstream NF1 sequences. Next, a modifying vector will be constructed that contains exon 23a along with a short length of surrounding intronic sequence and sequences homologous to portions of the NF1 homology vector. In addition, the modifying vector will contain the M1 mutations for increasing exon 23a inclusion (Fig. 2A), along with additional sequences that will be useful in later steps for deleting exon 23a and for distinguishing recombination products. The modifying vector will be recombined with the NF1 homology vector in E. coli to generate the NF1 targeting vector, which will be used in sub-aim B to introduce mutations into the genomic DNA of mouse ES cells by homologous recombination (Fig. 3C-H). To date, we have cloned the NF1 retrieval vector and are in the process of cloning the NF1 modifying vector (Fig. 3).

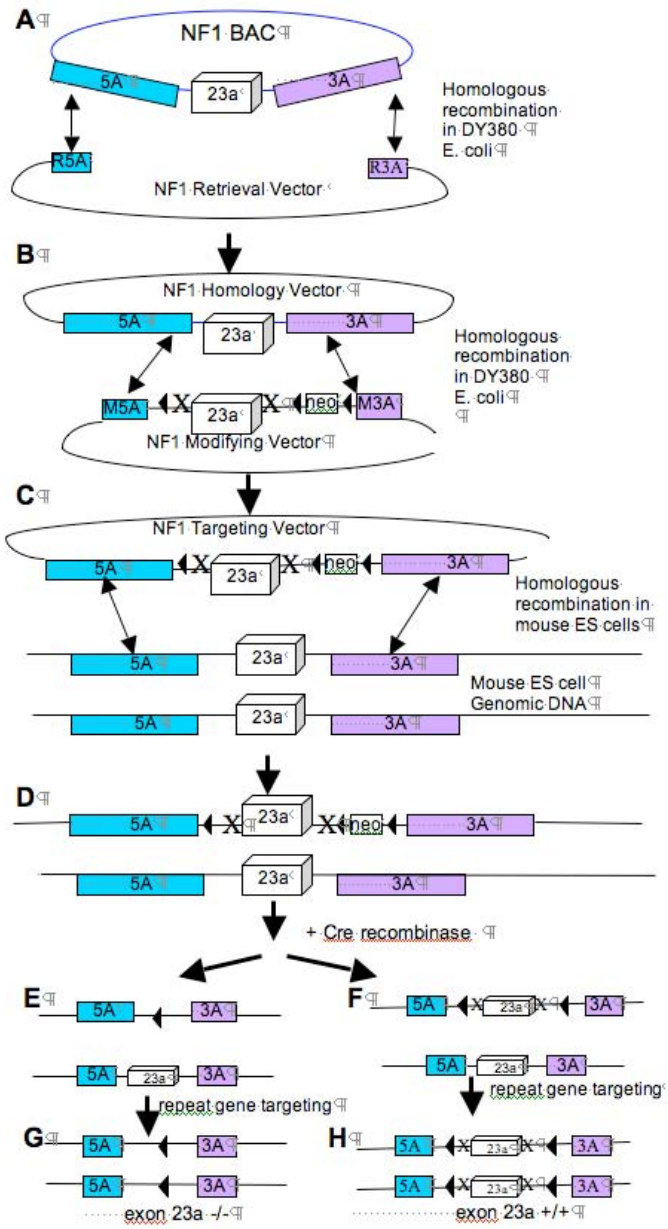

Figure 3. BAC modification and gene targeting. **A.** Recombination between NF1 BAC and NF1 retrieval vector to form NF1 homology vector. 5A and 3A: 5' and 3' homology arms; 23a: NF1 exon 23a; R5A and R3A: short sequences homologous to 5A and 3A. **B.** Formation of NF1 targeting vector by recombination between NF1 homology and modifying vectors. M5A and M3A: short sequences homologous to 5A and 3A; X: M3 mutations to increase exon 23a inclusion (see Fig. 5); neo: neomycin resistance; triangles: lox P sites. **C.** Gene targeting of mouse ES cell DNA by recombination with NF1 targeting vector. **D.** Gene targeting product with one DNA copy mutated. Cre-lox P recombination is induced to form products with neo removed and one copy of exon 23a either deleted **(E)** or mutated **(F)**. Gene targeting is repeated, forming ES cells with both copies of exon 23a deleted **(G)** or mutated to increase inclusion **(H)**.

KEY RESEARCH ACCOMPLISHMENTS

-Identified point mutations that will ensure predominant inclusion of the NF1 exon 23a.

-Made lentivirus expressing mHuB or mHuC.

-Designed the strategy for engineering the NF1 locus in mouse ES cells.

REPORTABLE OUTCOMES

Abstracts:

Melissa N. Hinman, Hui Zhu, Robert A. Hasman, Kavita Praveen, and Hua Lou. "Mammalian Hu protein family members have non-redundant functions as splicing suppressors". **Rustbelt RNA Meeting**, Deer Creek State Park and Resort, Mt. Sterling, OH, October 19-20, 2007. Poster presentation.

CONCLUSION

The results presented in this report laid the foundation for future studies. The reagents and tools generated from the past funding period will be very useful to carry out the next-step experiments towards the goal of the proposed studies. The most significant finding was that we were able to change the alternative exon 23a into a constitutive exon that can be included in most cell types by manipulating the splicing signals surrounding this exon. The implication for the NF1 disease is that if a mutation falls within these splicing signals, it may impact how this exon is spliced. Because this exon encodes part of the GAP-related domain, the potential biological outcome may be important. Obviously, this needs to be further investigated.

REFERENCES

None

APPENDICES

None